ABSTRACT

Tannase catalyses the hydrolysis reaction of the ester bond present in gallic acid esters and hydrosable tannins. This enzyme is produced by plants and microorganism and used in food industry such as tea industry. In order to produce high yields of tannase enzymes, it needs optimization of parameters which normally consuming a lot of time and expensive to be optimized conventionally. In this study, Response Surface Methodology (Central Composite Design) was performed for optimization. *Aspergillus niger* had been chosen as biomass and glucose as substrate in this study. The fermentation study was take place in the shake flask and was optimized for higher tannase enzyme activity. There are three factors was studied in this study which are the pH of the fermentation medium (pH 3.5-pH 6.0), the substrate concentration (2 w/v%-10 w/v%) and the agitation speed (100 rpm-300 rpm). From one factor at a time (OFAT) analysis, tannase enzyme showed that the optimum tannase activity at pH 5, 6.0 w/v% and 200 rpm with 14.9521 U/ml, 13.1256 U/ml and 12.4301 U/ml. RSM results shows that the optimum values of each parameter were pH 4.75, 6.0 v/w% of substrates concentration and 200 rpm of agitation speed which resulted the optimum tannase activity at 15.3131 U/ml. As a conclusion, RSM is the best tool used to identify the correlation between controlled independent factors and observed dependent responses. For the future research, it is recommended for study the application of genetic engineering in the enzyme production by scale up tannase production using a bioreactor and toxicology studies on tannase enzyme for application in food industry.

ABSTRAK

Tannase menjadi pemangkin tindak balas hidrolisis ikatan ester dalam ester asid gallic dan tannic hidrolisis. Enzim ini dihasilkan oleh tumbuh-tumbuhan dan mikroorganisma dan digunakan dalam industri makanan seperti industri teh. Dalam usaha untuk menghasilkan hasil enzim tannase yang tinggi, ia memerlukan pengoptimuman parameter yang biasanya mengambil banyak masa dan mahal untuk dioptimumkan konvensional. Dalam kajian ini, Kaedah Tindak Balas Permukaan (Reka Bentuk Komposit Berpusat) telah dijalankan untuk pengoptimuman. *Aspergillus niger* telah dipilih sebagai biomas dan glukosa sebagai substratu dalam kajian ini. Kajian fermentasi berlaku dalam kelalang goncang dan dioptimumkan untuk aktiviti enzim tannase yang lebih tinggi. Terdapat tiga faktor telah dikaji dalam kajian ini dimana pH untuk medium fermentasi (pH 3.5-pH 6), kepekatan substratu (2 w/v %-10 w/v %) dan kelajuan pengadukan (100 rpm-300 rpm). Dari analysis OFAT, enzim tannase menunjukkan bahawa aktiviti enzim tannase optimum pada pH 5, 6.0 w/v % kepekatan subsratu dan 200 rpm kelajuan pengadukan dengan 14.9521 U/ml, 13.2156 U/.ml dan 12.4301 U/ml. RSM menunjukkan bahawa nilai-nilai optimum parameter setiap pH 4.75, 6.0 w/v% kepekatan substratu dan 200 rpm kelajuan pengadukan yang mengakibatkan aktiviti tannase optimum pada 15.3131 U/ml. Sebagai kesimpulan, RSM adalah alat terbaik yang digunakan untuk mengenalpasti hubung kait antara factor bebas yang dikawal dan diperhatikan keputusan bergantung. Untuk penyelidikan pada masa akan dating, adalah disarankan untuk mengkaji aplikasi kejuruteraan genetik dalam penghasilan enzim, meningkatkan skala pengeluaran tannase dengan menggunakan bioreaktor dan kajian toksikologi enzim tannase untuk aplikasi dalam industry makanan.

TABLE OF CONTENTS

CHAPTER 5: CONCLUSION AND RECOMMENDATION

LIST OF TABLE

LIST OF FIGURE

LIST OF SYMBOLS

%	Percentage
$+\infty$	High factorial point
^0C	Degree Celsius
$-\infty$	Low level of factorial point
b_{ij}	Cross product coefficient
X	Cell dry weight
X_1	pH of samples
X_2	Substrate concentration of samples
X_3	Agitation speed of samples
Y	Tannase activity
ε	Error

LIST OF ABBREVIATIONS

% w/v	Percentage of weight over volume
$(NH_4)_3PO_4$	Ammonium Phosphate
Aa20	Strain number of *Aspergillus niger*
Adj R^2	Adjusted R^2
ANOVA	Analysis of variance
BSA	Bovine serum albumin
CCD	Central Composite Design
cm	Centimeter
DOE	Design of Experiment
et al.	An others
Exp	Experiment
$FeCl_3$	Ferum chloride
g	Gram
HCl	Hydrochloric Acid
Inc.	Incorporate
KH_2PO_4	Kalium dihydrogen phospate
M	Molar unit concentration
MARDI	Malaysian Agricultural Research and Development Institute
$MgSO_4.7H_2O$	Magnesium Sulphate Anhydrose
ml	milliliter
mL/min	Milliliter per minute
mm	Millimeter
NaCl	Sodium Chloride

NaOH	Sodium Hydroxides
OFAT	One factor at a Time
PDA	Potato Dextrose Agar
rpm	Revolution per minute
RSM	Response Surface Methodology
sp	Species
U	Unit of enzyme
U/g	Units of enzyme per gram
U/L	Units of enzyme per liter
U/L.h	Units of enzyme per liter per hour
Ver	Version
$ZnSO_4$	Zinc Sulfate

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF STUDY

Enzyme is proteins that occur in nature and increase the rate of the biochemical process. In the food industries, enzyme is used to produce everything like cheese to baked goods. This tannase enzyme application is used for the industry because it allows the manufacturer to produce more of a particular product in a shorter amount of time thus increasing the profit.

Nowadays, food industry represents one of the economic sectors by using the microbial in their application. In the field of biotechnology there are many industrial applications use to produce the biotechnological product that we use every day. Most of the enzyme has been produced by submerged culture at industrial level. Some of the food industries apply the enzyme to produce or make improvements in the quality of different foods. The microbial enzyme used as an aid processing in food industries (Belmares *et al.*, 2004)

Employment of the enzymes in food industry are the control of quality of certain foods , the modifications of the properties of some food additives and of the food itself and the production of enzymes used for food additives. In the wine industries, the enzymes are used for increasing the processing capacity and improve the economy (Belur *et al.*, 2010)

1.2 PROBLEM STATEMENT

Nowadays, the tannase enzyme is important to the food industry due to high demand in food and drink industry. There are many research had been done to develop the production of the extracellular tannase enzyme. But the production of the tannase enzyme is very low. Because of that, there are needs for an improved process for the production of tannase enzyme.

The economy problem was the major problem for the industry. The price for the enzyme is too expensive. So, by the ability from the microorganism to produce the enzyme, it can solve the economic problem to the industry.

There are a lot of microorganism can produce enzyme. So, the type of the microorganism is decided to be used in this study has become serious matter to observed. Because of that, after some review, it is decided to use the *Aspergillus sp.* from fungi because of their criteria to produce a lot of enzyme. It also easy to handle compared to bacteria. By addition of substrate like glucose to the culture medium at initial concentration, it will improve the production of enzyme tannase by *Aspergillus sp.*

For addition, the time is also being the problem in this study. Because of that, Response Surface Methodology is used in this study as it saves a lot of time. Moreover, from this method the optimum condition for enzyme production can be achieved.

1.3 OBJECTIVE

The objectives of this study are;

i. To produce extracellular tannase enzyme from the fermentation of *Aspergillus niger* using pure glucose.

1.4 SCOPE OF STUDY

To achieve the objectives, three scopes have been identified in this research:

i. To study the production of extracellular tannase enzyme in shakes flask fermentation using pure glucose as substrate for *Aspergillus niger.*
ii. To study the effects of pH controlling (3.5 – 6), the effects substrate concentration controlling (2 w/v% – 10 w/v%) and effects of agitation speed (100rpm – 300 rpm) to the extracellular tannase production in shake flask fermentation.
iii. To applied Response Surface Methodology using Design of Experimental software to design experimental work for extracellular tannase production.

1.5 RATIONALE AND SIGNIFICANCE

The aim of this study was to estimate optimum parameters values to get higher yield of extracellular tannase production in shake flask fermentation method by pure glucose as the media to *Aspergillus niger* culture.

In this study, the Response Surface Methodology (RSM), a central composite design of experiment is used to obtain the optimum parameter such as sucrose concentration, pH and agitation rate. This method is very important to the future study because it will save a lot of time during do the experiment. Besides, it also developed an equation to get the optimum for the production of the enzyme. Thus, Response Surface Methodology is more suitable and gives an advantage compare to the using one factor at a time.

As an addition, in this study, an extracellular tannase has been choosing. By this methodology, experimental works will easier compare to intracellular that are more costing, consumed a lot of time and tedious. Due to this reason, the extracellular is suitable in this study.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

A research had been done to identify cultivating conditions that influence tannase production by *Aspergillus sp.* The keywords that had been search function of tannase, microorganism that use to produce tannase, production of tannase, parameter used, and methods used to analysis tannase. This research is to find the best method, and parameter to increase the production of tannase.

2.2 TANNASE

Tannin acyl hydrolase or tannase catalyses the hydrolysis reaction of the ester bonds present in the hydrolysable tannins and gallic acid esters. Tannins are defined as naturally occurring water-soluble polyphenols of varying molecular weight depending on the bonds possessed with protein and polysaccharides (cellulose and protein) (Costa *et al.*, 2008). This enzyme is produced by plants part (bark, needles, heartwood, grasses, seeds, and flowers) and microorganism *(Aspergillus, Bacillus)*. The tannase enzyme catalysed the hydrolysis reaction of the ester bond present in the hydrolysable tannins such as tannic acid, methygallate, ethyl gallate, n-propylgallate, isomygallate, and releasing glucose and gallic acid. The tannase is mainly present in microorganisms *(Aspergillus, Bacillus)* even though it also present in plant and animal (Ayed and Hamdi, 2002; Nishitani and Osawa, 2003). Tannins are widespread in the plant kingdom, and found at different part at vascular plant such as bark, flowers, seeds and grasses (Belmares *et al.*, 2004).

Tannin are group of complex oligomeric chains substance that characterized by the presence of polyphenolic compounds. One of the major characteristic of the tannins is its ability to form strong complexes with protein and other macromolecules such as starch and cellulose and it also cause a reduction in nutrional value of food. There are two groups of tannins, hydrosable and condensed tannins (Lekha and Lonsane, 1997). A typical reaction of the tannase enzyme is hydrolytic cleavage of (-) epigallocatechin-3-ol gallate (Figure 2.1) (Bajpai and Patil, 1997; Lekha and Lonsane, 1997). The hydrolysable tannins are constituted by several molecules of organic, such as gallic, ellagic, digallic and chebullicacis, esterified to a molecule of glucose (Belmares *et al.*, 2004). Molecules with a core of quinic acid instead of glucose have been also considered as hydrolysable tannins, (Figure 2.1) presents some examples of hydrolysable tannins (Mueller-Harvey, 2001). In order to maintain their binding capacity, gallotannin or tannins must have more than two gallic acid constituent esterified to the glucose core. Hydrolysable tannins can be easier to hydrolysed under mild acid or alkaline conditions, with hot water or enzymatically (Lopez-Rios, 1984). Condensed tannis or Proanthocyanidins (Figure 2.2) are complex compounds that considered not being hydrolysable (Belmares *et al*., 2004). Their major constituted are cyaniding and delphinidin which responsible for astringent taste of fruit and wines (Sanchez, 2008). As mentioned before, tannase catalyzed the breakdown of hydrosable tannins such as tannic acid, methygallate, ethyl gallate, *n*-propylgallate and isoamygallate. A Figure 2.3 shows a typical reactions of tannase is the hydrolytic cleavage of (-) epigallocatechin-3-ol gallate (Bajpal and Patil, 1997; Lekha and Lonsane, 1997; Belmares *et al*., 2004).

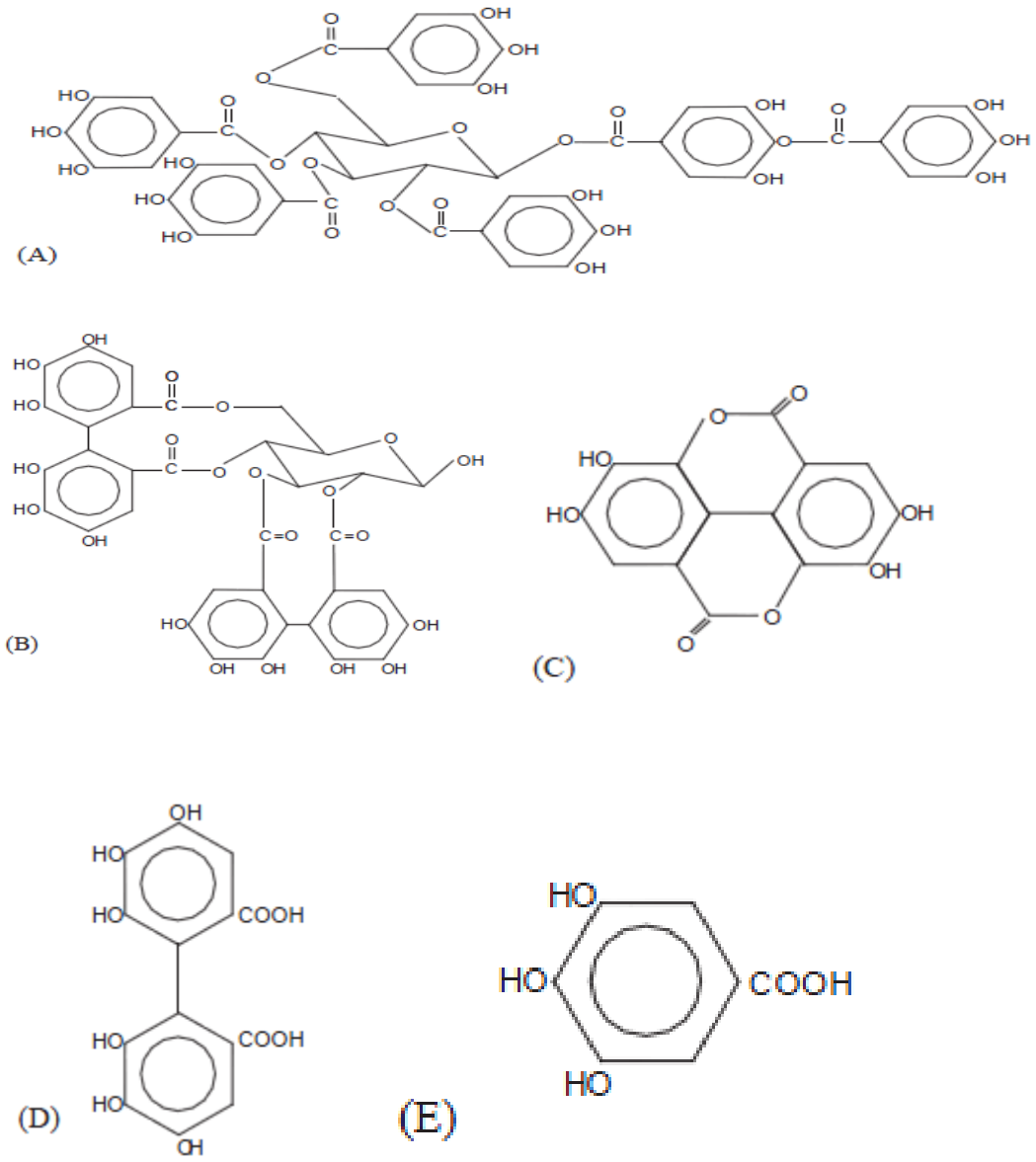

Figure 2.1: Hydrolysable tannins and some of their constituent, A. Gallotannin, B. Ellagitannin, C. Ellagic acid, D. Hexahydoxyphenic acid and E. Gallic acid.

Source: Mueller-Harvey, 2001

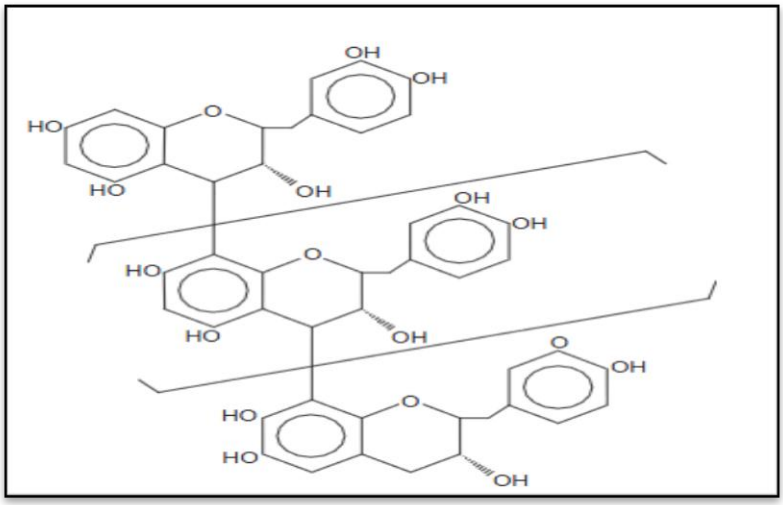

Figure 2.2: Condensed tannins or Proanthocyanidis.

Source: Belmares *et al.*, 2004

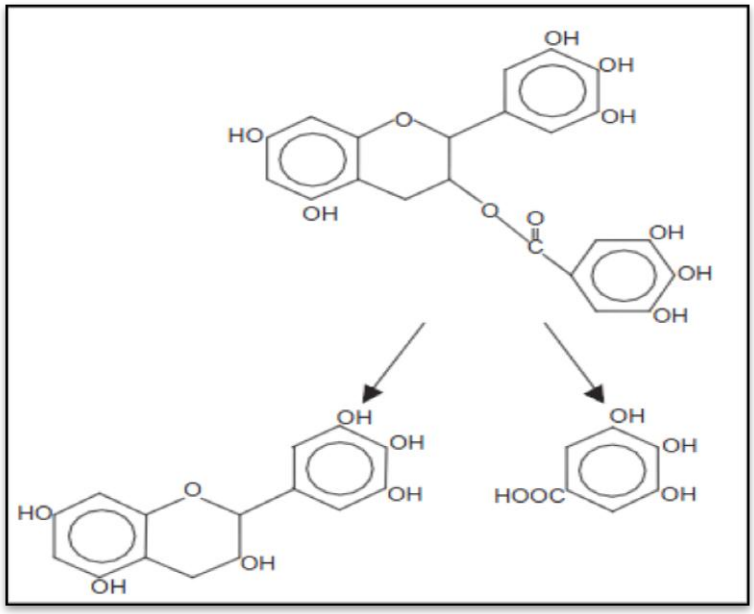

Figure 2.3: Typical reaction of tannase enzyme

Source: Belmares *et al.*, 2004

2.3 THE APPLICATION OF TANNASE ENZYME

Nowadays, the enzymes are commonly used in the industry such as chemical industry, food industry and many more according to their reactions. The advantages of using the enzyme are the mild reaction conditions, lower risk of toxic by-product, and their specificity (Aguilar *et al*., 2001). In the food manufacture, enzymes are mostly used as processing aids to improve the yield, texture, taste or other quality aspects. Enzyme tannase has also been expanding its uses in the global industry (Belmares *et al.*, 2004).

Other important application of tannase in the food industry is its uses as substrate for the chemical synthesis of pyrogallol or ester gallates, which are used as preservatives (Sharma and Gupta, 2003). Recently, tannase enzyme was commercialized in the hydrolysis of gallotannin to gallic acid that is important for the synthesis of propyl gallate in the food industry and it is an intermediate required for the synthesis of an anti-folic antibacterial drug trimethoprim. Gallic acid is extensively used as an ingredient of developer in photography and printing inks. As antioxidant gallic acid acts an anti-apoptotic agent and helps human cells against oxidative damage. Gallic acid also shows their cytotoxic activity against cancer cells, without harming normal cells (Bajpai and Patil, 2008).

This enzyme is extensively used in the food industry as clarifying agent such as preparation of instant tea, coffee, flavored soft drinks and also as additive for detannification of food (Lokeswari and Jaya Raju, 2007).

2.4 MICROORGANISM USED FOR TANNASE PRODUCTION

There are many microorganisms used for tannase production. Most of reported tannase production organisms are fungi and only a few bacteria (Lokeswari, 2010). For example, for bacteria are *Bacillus pumilus, Bacillus polymyxa, Corynebacterium sp, Klebsiella pneumonia, Streptococcus bovis,* and *Selenomonas remunantium,* for yeast are *Candida sp, Mycotorula japonica* and for fungi are *Aspergillus sp., Penicillium*

chrysegenum, Rhizopus oryzae, Trichoderma viride, Fusarium solani and *Mucor sp.* The production of tannase is depending on the strain and the culture conditions. It is showing different patterns for the different microorganisms. The filamentous fungi of the *Aspergillus* genus have been widely used for tannase production (Bajpal and Patil, 1996, Banerjee *et al*., 2001).

The production of the tannase enzyme by the *Aspergillus sp.* can occur in the absence of tannic acid, but these fungi tolerate tannic acid concentrations as high as 20% without having a deleterious effect on both the growth and enzyme production.The main advantage of producing fungi compared to bacterial cells is that they are typically much larger and are easily separated from the fermentation medium. (Belmares *et al.*, 2004)

Fungi also grow much more slowly than bacteria. A slower growth means that less nucleic acid is contained in the end product. In additions, fungi also have lysine content and ability to grow at acid pH. The disadvantages include lower growth rate, lower protein content and lower methionine content than in bacteria. For this research, the tannase produced by *Aspergillus niger* that have been carried out on submerged and solid state cultures. The addition of the carbon sources such as glucose to the culture medium, it will improve the production of tannase by *Aspergillus niger.* Table 2.1 show the microorganisms used for tannase production (Belmares *et al*., 2004).

Table 2.1: Microorganisms used for tannase production

Types of Microorganisms	
Bacteria	*Bacillus pumilus*
	Bacillus polymyxa
	Corynebacterium sp.
	Klebsiella pneumoniae.
	Streptococcus bovis
	Selenomonas rumunantium
Yeast	*Candida sp.*
	Saccharomyces cerevisiae.
	Mycotorula japonica
Fungi	*Aspergillus niger*
	Aspergillus oryzae
	Aspergillus japonicas
	Aspergillus gallonyces
	Aspergillus awamori
	Penicillum chrysogenum
	Rhizopus oryzae.
	Trichoderma viride
	Fusarium solani
	Mucor sp.

Source: Belmares *et al.*, 2004

2.5 *ASPERGILLUS NIGER*

Aspergillus niger in of the filamentous fungi that have been widely used for tannase production (Belmares *et al.*, 2004*). Aspergillus niger* is a filamentous fungus that give an important role in biotechnology. Table 2.2 shows the taxonomy of *Aspergillus niger.*

Table 2.2: Taxonomy of *Aspergillus niger*

Kingdom	Fungi
Phylum	Ascomycota
Class	Eurotiomycetes
Order	Eurotiales
Family	Trihocomaceae
Genus	*Aspergillus*
Species	*Aspergillus niger*

Sources: Universal Protein Resources, 2011

Aspergillus niger does have spores and reproduces asexually meaning that it can produce its offspring by themselves. According to Purwanto *et al.* (2009) research, they discovered that the morphology of *Aspergillus niger* using Scanning Electron Microscope (SEM). Meanwhile Rob Hoffmann (2010) identified the SEM of the asexual reproduction of *Aspergillus niger*.

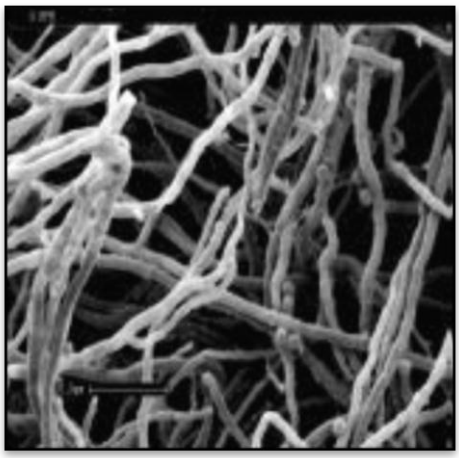

Figure 2.4: Filamentous structure of *Aspergillus niger*

Source: Purwanto *et al.*, 2009

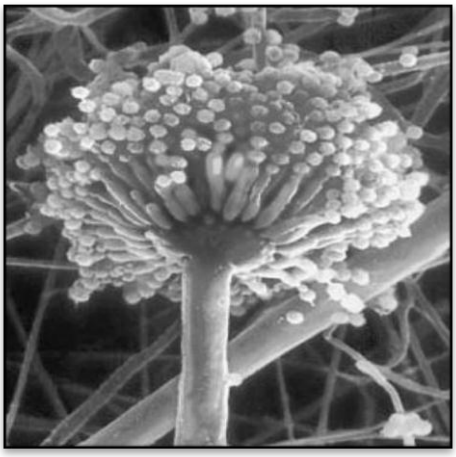

Figure 2.5: Asexual reproduction of *Aspergillus niger*

Source: Rob Hoffman, 2010

Filamentous fungi must have potential to grow under varying environmental conditions such fermentation time, pH, temperature and utilizing various sources of substrate such as nutrients (Ikram-Ul-Haq *et al.,* 2006). The tannase production can be produced by *Aspergillus niger* (Lokeswari and Jaya Raju, 2007), *Aspergillus oryzae* (Lokeswari, 2010), *Aspergillus tamari* (Costa *et al.,* 2008), and *Aspergillus awamori* (Beniwal and Chhokar, 2010).

2.6 PRODUCTION OF TANNASE ENZYME

The tannase enzyme production from *Aspergillus* can occur in absence of tannic acid, this fungi tolerates tannic acid concentration as high 20 percent without having a deleterious effect on both growth and enzyme production (Belmares *et al.,* 2004). The studies on tannase enzyme production by *Aspergillus sp.* can be done in various methods. There are various ways to produce the tannase from the microorganisms; they are liquid surface, submerging culture, modified solid-state cultures and solid state culture (Bradoo *et al.,* 1997, Belmares *et al.,* 2004).

The term of solid state fermentation (SFF) is applied for the processes in which insoluble material in water are used for the materials in water are used for the microbial growth (Aguilar *et al*., 2008). However, solid state fermentation is used to produce more than one enzyme and it variety, mainly from mold origin. This fermentative process

needs the water that does not exceed the capacity of the saturation of the solid bed in which the microbial grow. Water is essential for the microbial growth in the solid state fermentation and it present in thin layer and in occasions, absorbed inside these substrates (Aguilar *et al*., 2008).

The production of tannase by solid state culture has more advantage than submerged culture. The advantages of the solid state culture are it has the high production titles more than submerged culture, the extracellular nature of the enzymes and the stability to wide pH and temperature ranges (Lekha and Lonsane, 1994). Higher enzyme activities have been reached using solid state culture. The tannase activity also has maximum expressed intracellular in solid state culture 18 times more than submerge culture, while the extracellular activity in the solid state culture is 2.5 times more than submerge cultures. The solid state culture system minimizes the catabolic repression phenomenon. However, most of enzyme manufacture produces the enzyme using submerged culture or liquid surface fermentation techniques with enzyme filter of grams per liter (Belmares *et al*., 2004).

For submerged culture, some studies show that optimum production and regulatory aspects of tannase by moulds carried out in submerged culture. There are two major different are found about submerged culture and the solid state culture. That are the tannase yield production and productivity are higher in solid state culture than the submerged culture and the tannase location under solid state culture conditions in mostly extracellular, whilst it is bounded to the mycelium under the submerge conditions (Belmares *et al*., 2004).

From the research done by Aguilar *et al.* (2008) shows that the solid state culture is produce a lot of biomass yield compare to the submerge culture. The tolerance to high concentration of tannic acid by *Aspergillus niger* was lower in the submerge culture than in solid state culture. There are comparison between solid state culture and submerge culture. For solid state culture, the culture media are simple. Some substrate can be used directly as a solid media or enriched it with nutrient. Then, the products of interest are concentrated, that which facilitates its purification.

The quantity of waste generated is smaller than submerged culture. Then, it has low humidity content. The disadvantage of using solid state culture is that the microorganism growth was limited by levels of humidity. The determination of parameters such as humidity, pH, free oxygen and dioxide carbon, constitute a problem due to the lack of monitoring devices. And the scale up of solid state fermentation processes has been little studied and it presents several problems (Belmares *et al.*, 2004)

Different researcher use liquid surface fermentation, submerged fermentation and solid state fermentation for the tannase production. Among these, submerged fermentation process for tannase production is mostly preferred because the sterilization and process-control method are easier to engineer in this system (Paranthaman and Vidyalakshmi, 2009). Most of the tannase enzyme has been produced by submerged fermentation. Its production at industrial level is in a microbial way using submerged culture, where the activity is expressed mainly of intracellular form, implying additional costs in its production (Lekha and Lonsane, 1994). Depending on the strain and the culture conditions, the enzyme can be constitutive or inducible, showing different production patterns (Belmares *et al.*, 2004).

2.7 FACTOR EFFECTING THE PRODUCTION OF TANNASE ENZYME

According to Ikram-Ul-Haq *et al.* (2006), there are several conditions for the fungi to grow such as fermentation time, pH, temperature and utilizing various sources of substrate as nutrients. So, the effect of the parameters gives an influence to their tannase production.